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Transformation of *Acinetobacter* sp. Strain BD413(pFG4Δ*nptII*) with Transgenic Plant DNA in Soil Microcosms and Effects of Kanamycin on Selection of Transformants

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Here we show that horizontal transfer of DNA, extracted from transgenic sugar beets, to bacteria, based on homologous recombination, can occur in soil. Restoration of a 317-bp-deleted *nptII* gene in *Acinetobacter* sp. strain BD413(pFG4) cells incubated in sterile soil microcosms was detected after addition of nutrients and transgenic plant DNA encoding a functional *nptII* gene conferring bacterial kanamycin resistance. Selective effects of the addition of kanamycin on the population dynamics of *Acinetobacter* sp. cells in soil were found, and high concentrations of kanamycin reduced the CFU of *Acinetobacter* sp. cells from 10^9 CFU/g of soil to below detection. In contrast to a chromosomal *nptII*-encoded kanamycin resistance, the pFG4-generated resistance was found to be unstable over a 31-day incubation period in vitro.

Bacterial antibiotic resistance markers are the most frequently inserted genes in transgenic plants. However, the resistance genes do not encode desirable traits in commercially used plant varieties. Of the 15 different resistance genes incorporated into plants (17, 30), several encode resistance to clinically used antibiotics. Since plant DNA has been shown to persist in soil over extended periods of time (8, 25, 38, 39), concerns that these transgenes may spread horizontally to bacteria have been raised (17, 18, 22, 29). Sequence comparisons of genes isolated from wild plants and bacteria have indicated that horizontal gene transfer has occurred naturally between them (13, 32). Moreover, whole-genome analyses of bacteria suggest horizontal transfer of genetic material to be common and a major force in bacterial evolution (14, 40).

One mechanism of gene transfer that allows uptake of genetic material from diverged species in bacteria is natural transformation, which facilitates uptake of naked DNA in competent bacteria (15). Based on this mechanism, several laboratory studies have been conducted to elucidate the potential for plant-harbored resistance determinants to be taken up by naturally occurring bacterial recipients (2, 3, 21, 28). These studies have, however, not been able to demonstrate uptake of such determinants, nor have studies of bacteria obtained from soil samples from field trials with transgenic plants (8, 25). Detection of horizontal transfer in these studies relied upon the uptake of expressed and selectable genes in the bacterial recipients grown under optimized conditions or a positive DNA hybridization signal or PCR amplification of plant transgenes in the bacterial fraction of soil. However, direct analyses of DNA from soil samples often fail to demonstrate integration of plant transgenes into bacterial genomes. Transfer of smaller DNA fragments or nonexpressed or nonselected genes would rarely be detected in these studies.

Recently, uptake of transgenic plant-harbored DNA fragments by bacteria based on restoration of a partially deleted (10- or 317-bp internal deletion) bacterial kanamycin (KM) resistance gene (*nptII*) after recombination with transgenic plant-inserted homologues was demonstrated (5, 7). By exposing the naturally transformable bacterium *Acinetobacter* sp. strain BD413 to DNA isolated from transgenic sugar beet plants, these groups showed that the bacterium can access plant DNA under optimized in vitro conditions if homologous stretches of DNA are present. Studies done under optimized in vitro conditions often have little relevance to natural systems such as soil. For instance, agricultural soils are continually exposed to DNA from decaying plant material. In this study, we demonstrate that horizontal transfer of DNA isolated from transgenic sugar beet (*Beta vulgaris*) plants to bacteria, based on homologous recombination, can occur in sterile soil microcosms. Since the numbers of transformants generated in soil is expected to be very low, a selective advantage for their enrichment and, hence, environmental significance is needed. In this study we have therefore also determined the effects of increasing kanamycin concentrations on the population dynamics of transformant and recipient *Acinetobacter* sp. strain BD413 cells in soil and liquid soil suspension. In addition, the in vitro stability of the kanamycin-resistant bacterial phenotypes was determined over a 31-day period.

Recipient preparation and isolation of donor DNA. The gram-negative soil bacterium *Acinetobacter* sp. strain BD413 (pFG4) with a 317-bp deletion in the plasmid-harbored *nptII* gene was used as the recipient in all transformations (7, 11). The recipient inoculum was prepared as described by Nielsen et al. (19, 23). Portions of 100 μl (10^8 CFU per ml of water) were used for each filter and soil microcosm. For enumeration of CFU, aliquots were spread on solidified LB medium (19) supplemented with the antibiotics (all at 50 μg ml^{-1}) rifampin (chromosomally encoded resistance), ampicillin (pFG4 encoded resistance), and KM (pFG4-encoded resistance) for selection of transformants and incubated at 30°C for 3 days. Each experiment was repeated and done in triplicate or more on three to eight agar plates. Transgenic sugar beets containing a functional *nptII* gene (16) were used for purification of donor

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DNA. The transgenic sugar beet insert contained, from the left border of the T-DNA, the *bar* gene, the bidirectional promoter Tr1/Tr2, the *nptII* gene, the 3′ *ocs* terminator, the cAMV35S promoter, and the *cpBNYVV* gene (see references 7 and 21 for a further description of the transgenic plant material). DNA from conventionally grown sugar beets from adjacent field sites was used as a control. The plant DNA, isolated according to Trinker et al. (34), was reextracted with phenol-chloroform and chloroform and precipitated with isopropanol and ethanol before quantification on a UV spectrophotometer and DNA fluorimeter.

Transformations on filters and in soil microcosms. Filter and soil transformations were done as described in Nielsen et al. (19, 20). For filter transformation with purified transgenic plant DNA, 100 μl of a DNA solution (at concentrations of 10, 40, 80, and 160 μg DNA per 160-μl solution) was mixed with the bacterial inoculum. As controls, we used DNA from nontransgenic plants mixed with the inoculum and transgenic plant DNA only to check for sterility. A Flevo silt loam soil, sampled from microplots in Wageningen, The Netherlands, sterilized by gamma irradiation (4 megarads) with a ^{60}Co source (Gammaster BV, Ede, The Netherlands) was used for all microcosm studies (35). The sterile soil microcosms consisted of polypropylene cylinders of 1-cm^3 volume and 7 mm tall filled with 1.2 g (dry weight) of Flevo silt loam soil (see references 19 and 20 for a detailed description of the soil microcosms). After addition of the inoculum by careful distribution of the solution on top of the columns, the microcosms with the adsorbed bacterial suspension were incubated for 24 h before water or nutrients (100 μl) were administered similarly; purified DNA was added after a further 1-h incubation. In addition to water, two different nutrient solutions were used. Nutrient solution A has previously been described (20) and contained 2% lactic acid in addition to 5× M9 minimal medium salts with a 25× concentration of P salts. Solution B has been modified from A to include half-strength salt solution, different organic acids (0.2% each of acetate, lactate, citrate, tartrate, and succinate), and in addition the amino acids glutamic acid, proline, alanine, glycine, leucine, serine, arginine, glutamine, and valine at an amount corresponding to 25 times the concentration found per gram of maize rhizosphere soil (see reference 12). Following a 24-h transformation period at 20 or 30°C, the microcosms were sampled as described previously (20), and CFU were enumerated after a 72-h incubation period at 30°C. As controls, those described for the filter transformations were used and, in addition, transgenic sugar beet DNA was added to soil microcosms containing bacterial inoculum, nutrient-stimulated for 24 h at the time of plating, to check for the occurrence of plate transformants; no Km^r colonies were found in these experiments. Since sampling of bacterial cells was normally done at least 24 h after addition of DNA to the soil microcosms, our experimental procedures did not encourage transformants to occur during the plating procedure. Previous studies have shown that chromosomal DNA incubated in sterile soil for over 6 h was not available as a source of transforming DNA to competent *Acinetobacter* sp. strain BD413 cells (19, 23). PCR amplification spanning the partially deleted or repaired *nptII* gene on plasmid pFG4 was used to confirm the restoration of the *nptII* gene in restreaked transformants of *Acinetobacter* sp. cells. The sequences were amplified by the method described by Hofmann and Brian, (10) with primer set P1 and P2: P1 (1236), 5′ TGC TAA AGG AAG CGG AAC 3′; P2 (2929), 5′ AGG TCA ACA GGC GGT AAC 3′ (7). The primers were designed to amplify the Tn*5* region 1236 to 2929, which includes the *nptII* promoter, the complete *nptII* gene, and the *bleo* gene present on pFG4 in the *Acinetobacter* sp. cells. As described above, the transgenic sugar beet insert contained the *nptII* gene expressed with a different promoter and terminator region. PCR signals were not obtained from the transgenic plant material with these primers (data not shown). PCR conditions were applied as described previously (7, 23).

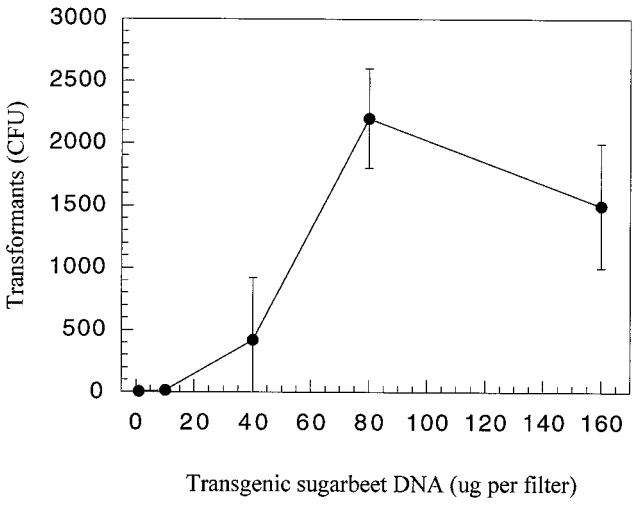

FIG. 1. Natural transformation and restoration of a 317-bp internal deletion in the *nptII* gene in *Acinetobacter* sp. strain BD413(pFG4) cells incubated on nitrocellulose filters with increasing concentrations of transgenic sugar beet DNA. T bars, standard deviations.

Selective effects of KM in soil and soil suspensions. For studies on the selective effects of KM in sterile soil, microcosms with inoculated Km^s (recipients) or Km^r (transformants) *Acinetobacter* cells (inoculated as described for the soil transformation studies) were treated with 100 μl of KM at concentrations of 0, 5, 12, 20, 50, and 100 mg ml of water^{-1}. After a further 24 h of incubation at room temperature, the microcosms were sampled as described for the soil transformation studies. To monitor the selective effects of KM in liquid soil suspensions, inoculated microcosms (see above) were suspended in 2 ml of LB medium with 1 g of gravel added ("aquarium sand," 2 to 4 mm in diameter, to aid in the suspension of soil particles) and the following antibiotics: rifampin, 100 μg ml^{-1}; ampicillin, 100 μg ml^{-1}; and 0, 10, 15, 20, 25, 50, and 100 mg of KM dissolved in 1 ml of water. The soil suspension was incubated at 27°C with shaking (180 rpm) for 24 h before sampling and enumeration of CFU. Numbers of replicates and repeats were as described for the soil transformations.

To determine the saturation level of transgenic plant DNA for transformation of *Acinetobacter* sp. strain BD413(pFG4) cells, increasing amounts of DNA were mixed with the recipient bacteria and incubated for 24 h on nitrocellulose filters (Millipore, Bedford, Mass.). As shown in Fig. 1, saturation for transformation was reached at a concentration of approximately 100 μg of DNA per 4×10^9 recipient CFU per filter. This DNA concentration, corresponding to the amount found in approximately 6.4 g of fresh plant leaves (producing 1 transformant per 2.9 mg of plant material or 11,600 copies of the *nptII* gene) (1, 5), was used for all the transformation studies in soil microcosms. de Vries and Wackernagel (5) obtained roughly the same numbers of transformants per microgram of plant DNA added when transforming *Acinetobacter* sp. strain BD413 cells in liquid cultures. However, they reported saturation of transformation to occur at a DNA concentration of 5 μg per 20 ml of liquid bacterial suspension. Since the number

of recipient CFU was similar in both studies (4×10^9 to 5×10^9), our 20-fold-higher number of transformants was also reflected in the correspondingly higher transformation frequency of 5.7×10^{-7} on solidified medium compared to that of 2.0×10^{-8} obtained in liquid culture (5).

As shown in Table 1, noncompetent (19, 20) *Acinetobacter* sp. strain BD413 cells residing in sterile soil microcosms for 24 h could, after addition of nutrients, be induced to integrate a bacterial marker gene inserted in transgenic sugar beets by homologous recombination. Both of the nutrient solutions used to stimulate competence development in *Acinetobacter* sp. strain BD413 populations contained inorganic salts and, in addition, simple organic compounds corresponding to those which have been frequently detected in the rhizosphere of various plants (4, 6, 12, 27). As seen from Table 1, solution B was efficient at inducing *Acinetobacter* sp. cells to higher transformation frequencies in soil microcosms. At 20°C, water or nutrient solution A was not able to promote transformation of the recipient with the transgenic sugar beet DNA. However, when solution A was provided to bacteria incubated in sterile soil for only 1 h, transformants were seen at a frequency of 2.2×10^{-8}. After 24 h of incubation in soil microcosms at a temperature of 30°C, both nutrient solutions A and B were able to generate transformants of *Acinetobacter* sp. cells (Table 1), producing up to 1.4×10^{-8} transformants per recipient, which corresponds to 1.2×10^{-7} transformants per plant-harbored copy of the *nptII* gene. Water was not able to induce any transformants in our studies, nor was nontransgenic plant DNA, soil microcosms with only nutrients and inoculum added, or soil microcosms with nutrient-stimulated bacteria and transgenic sugar beet DNA added at the time of sampling. The latter control indicated that transformation did not occur on the selective plates. The restoration of the *nptII* gene in some of these randomly picked colonies was revealed by PCR amplification (Fig. 2). Inoculation of GN-Biolog plates, quantified on a Biolog MicroLog station (Biolog Micrologg3; Biolog, Inc, Hayward, Calif.), with transformants also revealed that their metabolic pattern was indistinguishable from the one obtained from the inoculant strain.

Transformation of *Acinetobacter* sp. strain BD413(pFG4) cells with transgenic sugar beet DNA was not detected in nonsterile soil microcosms (data not shown). In previous gene transfer studies with homologous bacterial DNA in sterile and nonsterile soil microcosms, a 10- to above 100-fold drop in the transformation frequencies has been observed with the change from sterile to nonsterile soil conditions (19). Thus, we infer that the transformation frequencies of *Acinetobacter* sp. cells in nonsterile soil microcosms, given similar purified transgenic plant DNA and nutrient conditions, would be at a level of 10^{-10} to 10^{-11} transformants per recipient. Transformations occurring with this efficiency were below our limit of detection (Table 1), and the identification of such events in nonsterile soil, using the same experimental setup as for the sterile soil, would require screening of approximately 20,000 agar plates. We emphasize that the estimates proposed here are based upon integration of the transgenic plant-harbored marker genes into the bacterial chromosome after homologous recombination, using purified transgenic plant DNA. Experimental studies by our groups (21) and others (2, 3, 28) have confirmed the low probability of integration of transgenes in recipient bacteria if DNA homology is not present. Bacterial genes and vector sequences are however present in most transgenic plants (see Table 3 in reference 22). Strictly homology-based recombination of plant transgenes in competent bacteria is unlikely to cause any environmental effect due to the already existing homologues in the bacterial chromosomes; however,

TABLE 1. Natural transformation and restoration of a 317-bp-deleted *nptII* gene in *Acinetobacter* sp. strain BD413(pFG4) cells residing in a sterile silt loam soil microcosm for 24 h, with added purified DNA from transgenic sugar beet (*B. vulgaris*) with a functional *nptII* gene

Addition[b]	20°C soil temperature[a]				30°C soil temperature[a]			
	Transformants (CFU)	Recipients (CFU)	Transformation frequency[c]	Transformants/*nptII* copy	Transformants (CFU)	Recipients (CFU)	Transformation frequency[c]	Transformants/*nptII* copy
Water	nd[d]	$9.5\ (1.2)^e \times 10^7$	$<1.1 \times 10^{-8}$					
Nutrients A	nd	$1.8\ (0.1) \times 10^8$	$<5.6 \times 10^{-9}$		nd	$1.2\ (0.1) \times 10^8$	$<8.3 \times 10^{-9}$	
Nutrients B	3.1 (3.4)	$2.1\ (0.1) \times 10^8$	1.4×10^{-8}	$9.8\ (10.7) \times 10^{-8}$	2.4 (3.8)	$2.1\ (0.2) \times 10^8$	1.2×10^{-8}	$7.6\ (11.9) \times 10^{-8}$
					3.7 (3.8)	$2.7\ (0.2) \times 10^8$	1.4×10^{-8}	$1.2\ (11.9) \times 10^{-7}$

[a] Following 24 h of bacterial presence in soil, nutrients and plant DNA (after 1 h) were added, and after another 24 h at 20 or 30°C, CFU were determined.

[b] Solution A, 5× M9 salts with 25× P salts and 2% lactic acid (20). Solution B, 2.5× M9 salts with 12.5× P salts and 0.2% solutions of lactate, succinate, acetate, citrate, and tartrate, corresponding to the concentration of organic acids found in the maize rhizosphere, and the amino acids glutamic acid proline, alanine, glycine, leucine, serine, arginine, glutamine, and valine corresponding to 25 times the concentration of amino acids found per gram of maize rhizosphere soil (12).

[c] Transformation frequencies are given as the number of CFU growing on transformant-selective LB agar plates divided by the number of CFU on recipient-selective plates. The data are presented as mean values for triplicate experiments ± standard deviations. The limit of detection is given as the reciprocal of the number of recipient cells.

[d] nd, not detected.

[e] Standard deviations (±) are given in parentheses.

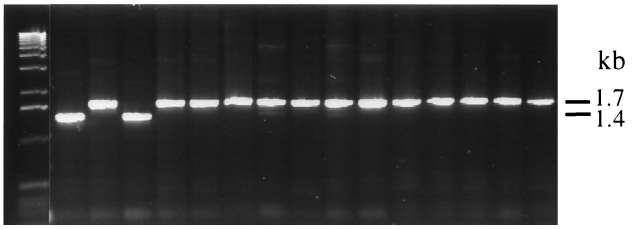

FIG. 2. PCR amplification of the Tn*5* region 1236 to 2929, which includes the *nptII* promoter, the complete *nptII* gene, and the *bleo* gene of pFG4, demonstrating the presence of the restored *nptII* gene construct in the transformant colonies and the 317-bp partially deleted gene in the recipient bacterium. Lane 1 from the left side, 1-kb ladder (Gibco-BRL); lane 2, *Acinetobacter* sp. strain BD413(pFG4) Km^s; lane 3, *Acinetobacter* sp. strain BD413(pFG4) Km^r; lane 4, *Acinetobacter* sp. strain BD413(pFG4) Km^s recipient; lane 5 to 16, *Acinetobacter* sp. strain BD413(pFG4) Km^r transformants obtained with transgenic *nptII*-containing sugar beet DNA.

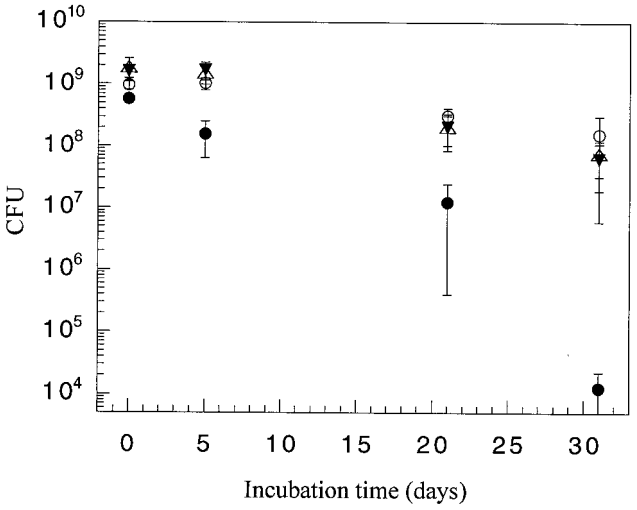

FIG. 3. Stability of KM-resistant phenotypes of *Acinetobacter* sp. strain BD413(pFG4::*nptII*) (circles) and *Acinetobacter* sp. strain BD413 *chr::nptII* (triangles) in liquid LB medium measured after plating on KM-free (open symbols) and KM-containing (filled symbols) LB medium. T bars, standard deviations. CFU are per 5 ml.

studies of gene transfer by natural transformation in competent bacteria have revealed that additive integration of nonhomologous genetic material can occur at high frequencies when flanking homology is present. For instance, in *Acinetobacter* sp. cells, uptake and integration, by natural transformation, of three nonhomologous foreign genes (*nptII*, *cryIVb*, and *aadB*) occurs, when flanking homologous bacterial DNA is present, at frequencies (transformants per recipient) up to 1% in vitro and 10^{-5} in nonsterile soil (19, 20). Thus, the presence in transgenic plants of various prokaryotic markers and vector sequences may facilitate additive insertion of foreign genetic material into bacterial hosts after homology-based heteroduplex formation.

Due to the expected low level of formation of transformants in soil, random genetic drift and selection (see below) may contribute to their possible amplification and hence environmental significance. Stability of the acquired resistance trait would be a prerequisite for random genetic drift to fixation. Stability in vitro of the Km^r phenotype in *Acinetobacter* sp. strain BD413(pFG4) bearing restored KM resistance was compared to the stability of the Km^r phenotype of *Acinetobacter* sp. strain BD413 with a chromosomally inserted *nptII* gene (19). Both resistant strains were inoculated (six replicates) in separate aliquots of 5 ml of nonselective LB medium and incubated at 27°C with agitation (180 rpm). The aliquots were sampled after 0, 5, 21, and 31 days on LB plates with or without KM (50 mg/liter), and CFU were enumerated after 72 h. As shown in Fig. 3, the Km^r phenotype in the strain harboring a chromosomally inserted *nptII* gene was stable, without selection, once the resistance trait had been acquired. The Km^r phenotype in the strain harboring the restored pFG4 plasmid was in contrast unstable and resistance was partially lost during the long-term incubation. The loss of KM resistance could be shown to be accompanied by a loss of the plasmid-encoded ampicillin resistance, suggesting that the altered Km^s phenotype seen was caused by plasmid instability.

KM-resistant bacteria, but not *nptII*-encoded phenotypes (31), are abundant in natural soils (found at levels of 10^5 CFU/g of soil), suggesting a possible selection of this phenotype in the soil environment. Despite this observation, the natural occurrence of antibiotics, like KM, in soil has been difficult to quantify, and the selective effects and role of antibiotics in soil remain unclear (24, 33, 36, 37). To our knowledge, no studies have detected KM in natural soil, and the antibiotic has been suggested to be inactivated in soil due to binding to clay minerals (9). Putative selective effects of KM on Km^s (recipients) or Km^r (transformants) *Acinetobacter* sp. cells in sterile soil microcosms were investigated in this study by administering increasing amounts of the antibiotic to the inoculated microcosms. As seen from Fig. 4a, the addition of up to 8.3 mg of KM (per g [dry weight] of soil) did not substantially influence the survival of Km^r *Acinetobacter* sp. cells in soil. In contrast, the Km^s cells were susceptible to the selective effects of the KM. However, only high amounts of KM reduced the number of recipients substantially over a 24-h period. The addition of up to 4.2 mg of KM reduced the CFU numbers less than fivefold, whereas a 1,100-fold drop of the recipient CFU was seen when 8.3 mg of KM was added. The latter addition is close to the upper limit of administering liquid KM in soil and equals 100 μl of a 100-mg ml^{-1} KM stock solution per 1.2 g (dry weight) of soil. As seen from Fig. 4a, 8×10^4 CFU of Km^s bacteria were still present in soil after this KM amendment and a 24-h selection period. To reveal if it was possible to obtain stronger selective effects of KM on soil bacteria, the microcosms were suspended in a total of 3 ml of liquid medium. This facilitated the addition of 10-fold-higher concentrations of KM to the bacterial suspension. Although negative selective effects of this high KM amendment were seen for the Km^r transformants, with a 540-fold reduction in CFU from 0 to 42 mg of KM added per g of dry soil (Fig. 4b), the same 42-mg addition of KM to the soil suspension with Km^s bacteria reduced their CFU from 4.6×10^9 to below detection over a 24-h period of selection. Reactivation of the selective effects of KM, inactivated after distribution and incubation in soil microcosms, after suspension of the soil in liquid culture was not seen (data not presented). Oliveira et al. (24) investigated the effects of KM amendment in soil microcosms on detection of Tn*5* (*nptII*)-carrying *Pseudomonas fluorescens*. Addition of 0.018 or 0.18 mg of KM g of dry soil^{-1} had no noticeable effect on the survival of the inoculated bacteria. Thus, both studies confirm that the addition of high levels of KM does not substantially influence the population dynamics of resistance-carrying bacteria in different soil microcosms. In contrast to Oliveira et al. (24) we were able to see effects of the added antibiotic on KM-sensitive bacteria after a 24-h incubation period in sterile soil, but only after high levels of antibiotic amendment: 8 to 42

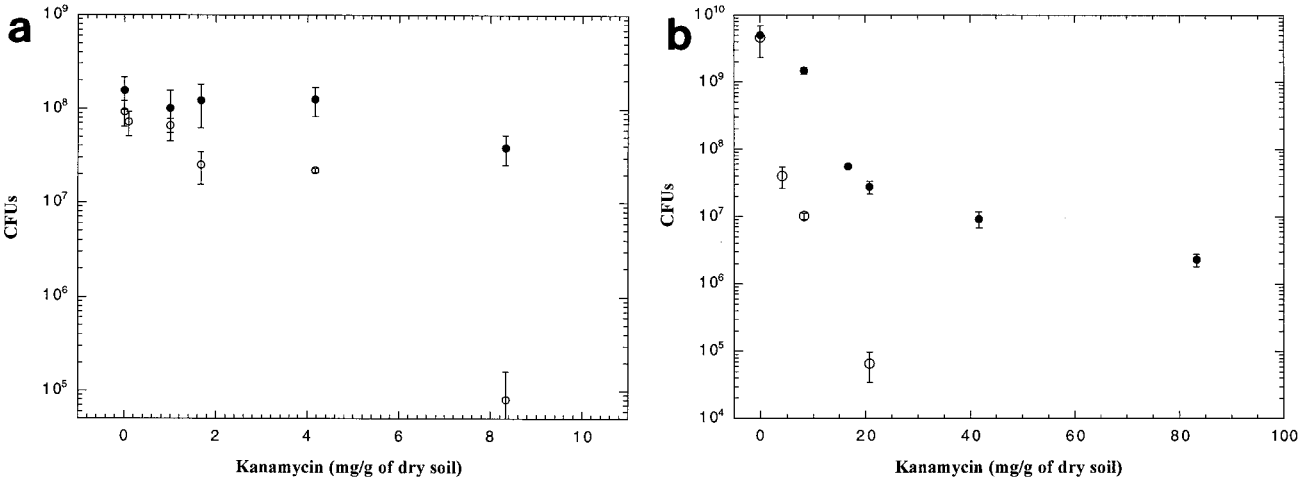

FIG. 4. CFU of KM-sensitive recipients (open symbols) and KM-resistant transformants (filled symbols) of *Acinetobacter* sp. strain BD413(pFG4) isolated from soil after addition of increasing amounts of kanamycin to soil microcosms (a) or liquid soil suspensions (b). T bars, standard deviations. CFU are per microcosm of 1.2 g (dry weight) of soil.

mg of KM g of soil^{-1}, corresponding to a 1,000-fold-higher concentration than normal amendment in bacterial media.

Based on the above studies with sterile soil microcosms regarding the effects of KM amendment on the population dynamics of *Acinetobacter* sp. cells, the earlier reported rapid KM inactivation by clays, and the apparent low presence of KM in soil, we suggest that natural soil conditions rarely would produce the selective pressure required for fixation of possible transfers of the *nptII* gene from transgenic plants into the recipient bacterium studied. However, we note that KM is able to select for different phenotypes in sterile soil microcosms. Furthermore, based on studies using purified DNA and sterile soil conditions, we indicate that homologous recombination, and possible additive integration, of bacterial marker genes harbored in transgenic plants into competent soil bacteria like *Acinetobacter* spp. may take place in soil and that the environmental significance of such rare events depends upon selection of the acquired character.

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